Revised Version of Manuscript

Submitted to Acta Biomaterialia

on January 2016

Special Issue on Zwitterionic Materials

Zwitterionic Ceramics for Biomedical Applications

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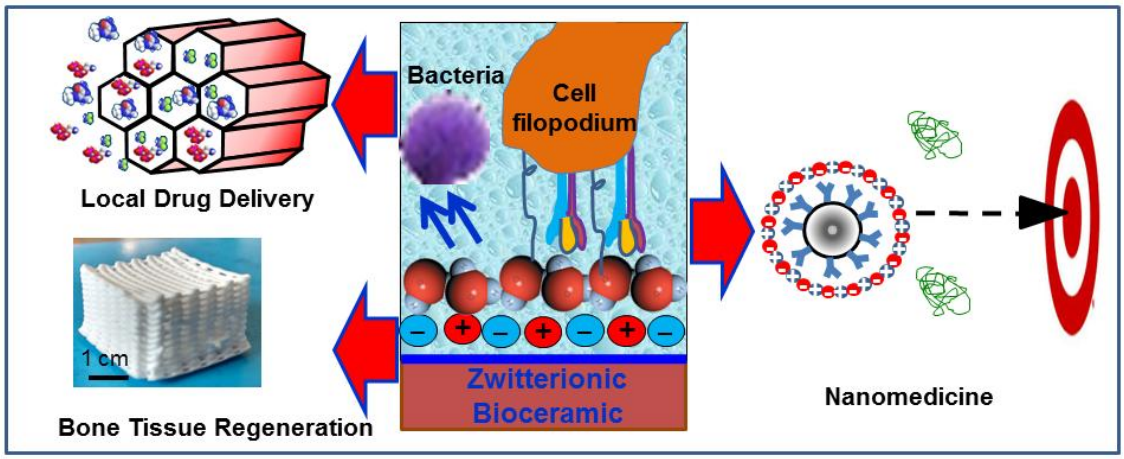
Abstract

Bioceramics for bone tissue regeneration, local drug delivery and nanomedicine, are receiving growing attention by the biomaterials scientific community. The design of bioceramics with improved surface properties able to overcome clinical issues is a great scientific challenge. *Zwitterionization* of surfaces has arisen as a powerful alternative in the design of biocompatible bioceramics capable to inhibit bacterial and non-specific protein adsorption, which opens up new insights into the biomedical applications of these materials. This manuscript reviews the different approaches reported up to date for the synthesis and characterization of zwitterionic bioceramics with potential clinical applications.

Graphical Abstract



Keywords: Zwitterionic bioceramics, functionalization, characterization, local drug delivery, bone tissue regeneration, nanomedicine.

1. Introduction

Bioceramics have experienced great development in the last 50 years [1]. Their biomedical applications include bone tissue regeneration, local drug delivery and nanomedicine, which are receiving increasing attention by the biomaterials scientific community [2-6]. The custom-made design of bioceramics attending to their envisioned clinical application constitutes a great scientific challenge. Bioceramics for bone tissue regeneration and local drug delivery must fulfill some specific requirements such as allowing adequate biocompatibility and cell response. Infection constitutes one of the major concerns in these biomedical fields, with high morbidity and costs to the national healthcare systems [7]. The design and development of bioceramic surfaces capable to inhibit bacterial adhesion meanwhile allowing osteoblast cells colonization would be and added value to achieve better clinical outcomes [2].

On the other hand, bioceramic nanoparticles (NPs) provide many opportunities in the nanomedicine landscape, such as therapy and diagnosis [6]. One of the main drawbacks that NPs have to face once they are in contact with physiological fluids is their trend to associate to plasma proteins, forming a "protein corona" that may lead to their clearance from the bloodstream or interfere in their final fate. In this sense, the design of bioceramic NPs with non-fouling properties would constitute a step forward in their potential applications. One of the most widely used strategies consists in grafting polyethylenglycol (PEG) onto the surface of these NPs. Nonetheless, this method usually provokes an increase in the overall hydrodynamic NP size, which could alter the *in vivo* final destination of these nanosystems [8].

Zwitterionization has emerged as a powerful tool to provide bioceramic surfaces of antibacterial adhesion and resistance to non-specific protein adsorption capabilities. Zwitterionic materials are characterized by owning equal number of both positively and

negatively charged groups onto their surface thus maintaining overall electrical neutrality [9]. The non-fouling capability of zwitterionic materials is related to a hydration layer onto the surface, since a tightly bound water layer forms a physical and energetic barrier that prevents bacterial adhesion and non-specific protein adsorption. It is possible to optimize functionalization methods to graft both positively and negatively charged moieties onto the bioceramic surfaces. Thus, different *zwitterionization* approaches have been developed so far to prepare advanced zwitterionic bioceramics.

Herein the main strategies reported to date for the *zwitterionization* of bioceramics are overviewed. An overall discussion of the different mechanisms that govern the colonization of biomaterial surfaces by cells and bacteria is also presented. In addition, the diverse characterization techniques to evaluate the zwitterionic nature of these materials are described. Finally, the different biomedical applications of zwitterionic bioceramics, focusing on bone tissue regeneration, local drug delivery and nanomedicine are reviewed.

2. Interactions between Bioceramic Surfaces and Biological Milieu

Recently, *zwitterionization* of bioceramics has emerged as a groundbreaking strategy to confer surfaces of high resistance to nonspecific protein adsorption, bacterial adhesion and/or biofilm formation [9]. The biocompatibility of bioceramics is very closely related to cell behavior in contact with them and particularly to cell adhesion to their surface. The surface characteristics of these materials as their topography, chemistry or surface energy, play an essential role in their biocompatibility [2]. It is important to remark that depending of the bioceramic functionality the specific requirements in terms of cells/proteins adhesion are completely different. Thus, for bone tissue regeneration purposes tailoring the surfaces of implants to actively promote bone bonding while

avoiding bacterial colonization represents an interesting challenge to achieve better clinical results. This phenomenon can be achieved through a double approach: the antibacterial properties of the implant surface itself and the capability to promote eukaryotic cell integration, *i.e.* the "race for the surface" theory. This concept, which was introduced in 1987 by the orthopedic surgeon Gristina [10], contemplates a race between eukaryotic cells and bacteria for the biomaterial surface. Thus, the real goal is to design implant surfaces that make the race being won by eukaryotic cells. This would allow the biomaterial surface to be covered by cell tissue, which will make it less susceptible to bacterial colonization. However, these requirements are totally different for NPs as drug nanocarriers or contrast agents in nanomedicine, since the major problem is their opsonization and scavenging by the mononuclear phagocytic system (MPS), which demands non-fouling surfaces able to totally inhibit serum protein adhesion.

Basically, the way eukaryotic cells interact with a surface is through an interface which consists of discrete attachment points denoted as integrins (see Figure 1A). These integrins interact with specific moeties of the extracellular matrix, such as RGD motifs [11]. In this sense, the surface chemistry at the nanoscale can be a deciding factor in which type of integrin will mediate cell adsorption onto the biomaterial surface [12]. On the contrary, bacteria are prokaryotic cells and differ from eukaryotic cells in two main aspects [13]: *i)* their cell wall is composed of phospholipids, like eukaryotic cells, but they are much more rigid due to an external layer of peptidoglycan, and *ii)* they are much smaller in size than eukaryotic cells (see schematic depiction in Figure 1A). It has been demonstrated that both features of bacteria, smaller size and stiffness than eukaryotic cells, might be key factors accounting for their capability to perceive variations in the chemical and topological features of biomaterial surfaces at the

nanoscale [2]. In terms of surface adhesion, whereas single eukaryotic cells can adhere to surfaces independently of each other through integrins, bacteria live on surfaces as a community within a resistant-extracellular polymeric substance, denoted as bacteria *biofilm*. The first step of *biofilm* formation, *i.e.* adhesion, is governed primarily by the electrostatic attraction forces between bacteria and surfaces (mediated by adhesins) and where the electrochemical nature of the bioceramic plays a major role [14,15]. Therefore, zwitterionic surfaces are capable of forming a hydration layer that would act as barrier to hinder bacterial adhesion, which would allow preventing infection in the bioceramic. In fact, recently, opposite behavior against bacterial and osteoblast cells in different bioceramics with zwitterionic nature has been described [16,17]. These results demonstrate that bioceramics could be specifically designed to promote osteoblast function while reducing bacterial colonization, which would be particularly pertinent for the successfully fabrication of bone implants. Similar results have been also described for nanostructured surfaces of Ti6Al4V implants [18]. However, the mechanisms regulating the bacterial and cells response to nanostructured surfaces have not been fully elucidated, although they seem to be mainly based on the smaller size and stiffness of the bacteria able to sense entities in the nanoscale [19].

On the other hand, regarding NPs for application in nanomedicine, when NPs contact physiological fluids they are prone to experience non-specific adsorption of biological molecules, mainly plasma serum proteins, which creates a dynamic "protein corona" (*opsonization*) that makes difficult to foresee and/or regulate their *in vivo* behavior (Figure 1B) [20,21]. In fact, formation of "protein corona" followed by recognition and engulfing by the cells of the MPS is a common final destination of many NPs, which are rapidly cleared from the bloodstream [22,23]. Thus, for such applications it is essential to avoid the serum protein adhesion by designing nonfouling surfaces with "stealth"

properties during their path in the bloodstream to reach the target cell and/or tissue and to exert the desired functionality.

3. Chemical Strategies for the *Zwitterionization* of Bioceramics

3.1. Functionalization with Low-Molecular Weight Zwitterionic Moieties

It is possible to functionalize the surface of bioceramics by grafting low-molecular weight zwitterionic moieties. This strategy offers several advantages compared to those involving zwitterionic polymers, because it usually involves more simple and "user-friendly" methods and, in the case of NPs, this method does not increase their hydrodynamic size. Herein we summarize the recent approaches to graft amino acids, sulfobetaine (SB) derivatives, or alkoxysilanes, onto the surface of bioceramics.

3.1.1. Amino Acids

Amino acids are good alternatives to provide bioceramics of zwitterionic nature. For instance, cysteine (Cys), which is a low cost and widely available amino acid, was grafted to the surface of silica NPs via a two-step procedure [24]. In a first step the surface silanol groups of silica NPs were reacted with an epoxide-containing alkoxysilane in an acidic water/ethanol mixture. Then, a coupling reaction between epoxide surface groups and free sulfhydryl group of Cys was carried out under basic pH conditions, yielding a thioether linkage (structure **1**, Figure 2).

3.1.2. Sulfobetaine Derivatives

Schlenoff and co-workers reported the grafting of the highly water-soluble SB siloxane (SBS), to the surface of silica NPs (structure **2**, Figure 2) [25,26]. In a first step they synthetized and isolated SBS by ring opening addition of propane sultone by an aminoalkylsiloxane. Then, SBS was added to the silica NPs in different amounts to

determine the minimum surface coverage needed to confer stability on the particles in salt and protein media. The same authors reported the functionalization of superparamagnetic iron oxide (γ–Fe_2O_3) NPs (SPIONPs) by using SBS by either *in situ* or post-synthesis *zwitterionization* (structure **2**, Figure 2) [27].

On the other hand, dopamine (DOPA) has been reported to have a high affinity for IONPs due to strong chelation of the catechol unit to the iron centers. This has been exploited to develop 10 nm-sized zwitterionic SPIONPs sing a compact DOPA sulfonate ligand (structure **3**, Figure 2) [28-30].

3.1.3. Mixtures of Alkoxysilanes

It is also possible to exploit silanes chemistry to graft alkoxysilanes bearing charged organic groups onto the surface of hydroxyl(–OH)-containing bioceramics. There are two main strategies, the co-condensation and the post-synthesis method. Co-condensation route, which has been used during the synthesis of silica-based mesoporous materials (SMMs), involves the addition of alkoxysilanes together with the silica precursor (typically tetraethylorthosilicate, TEOS) during the synthesis step. Thus, SBA-15-type mesoporous silica bearing $–NH_3^{\oplus}/–COO^{\ominus}$ groups was prepared by using stoichiometric amounts of 3-aminopropyltrimethoxysilane (APTES) and carboxyethyl silanetriol sodium salt (CES) organosilanes as ammonium and carboxylate sources, respectively, during the synthesis (structure **4**, Figure 4) [16,31]. Following this strategy, zwitterionic SBA-15 was also prepared by using an alkoxysilane containing primary and secondary amine groups (N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane) (DAMO) (structure **5**, Figure 2) [32]. The zwitterionic nature of this material comes from the $–NH_3^{\oplus}/–SiO^{\ominus}$ and $>NH_2^{\oplus}/–SiO^{\ominus}$ zwitterionic pairs present onto the material surface.

The post-synthesis method consists in covalently grafting suitable organosilanes to the –OH groups of as-synthesized bioceramics. Accordingly, the P–OH groups present in the surface of hydroxyapatite (HA) were reacted under anhydrous conditions with a mixture of APTES and CES, used as $–NH_3^{\oplus}$ and $–COO^{\ominus}$ sources, respectively (structure **4**, Figure 4) [17].

3.2. Functionalization with Zwitterionic Polymers

Zwitterionic polymers, with mixed positively and negatively charged moieties within the same polymer chain and overall charge neutrality, have been widely used to design ultralow fouling surfaces able to resist nonspecific protein adsorption, bacterial adhesion and biofilm formation [33-37]. The most widely used method to graft zwitterionic polymers to bioceramic surfaces is the surface-initiated atom transfer radical polymerization (SI-ATRP) [38]. This method has been used to coat silica NPs with poly(sulfobetaine) (pSB) (structure **6**, Figure 2) [39] and polycarboxybetaine (pCB) derivatives [40,41]. Alternatively, surface reversible addition-fragmentation chain transfer (RAFT) polymerization has been used to graft SB copolymers from mesoporous silica nanoparticles (MSNPs) [42]. Although "graft-from-surface" methods have been widely exploited, they require multistep synthetic procedures and very careful conditions which limit its practical application. To address this problem, another strategy, the "graft-to-surface" method has been developed. In this method, polymers carrying adhesive moieties with strong surface affinity are synthesized and then grafted onto the surface through their adhesive moieties. Using this approach, IONPs were coated with pCB derivatives via two 3,4-dihydroxyphenyl-L-alanine-DOPA surface adhesive groups (structure **7**, Figure 2) [43]. More recently, Xiao *et al.* have developed an easy route to introduce zwitterionic nature to poly(acrylic acid) (pAA)-coated IONPs

by using EDC/NHS chemistry to couple 3-(diethylamino)propylamine (DEAPA) to a significant fraction of the surface carboxyl groups (structure **8**, Figure 2) [44].

4. Methods for the Characterization of Zwitterionic Materials

Chemical characterization of zwitterionic biceramics is crucial to predict and understand the performance of these materials in biological milieu. For this purpose, the different characterization techniques must fulfill some requirements such as *i)* getting information about the physicochemical nature of the surface at the nanoscale and/or atomic level; *ii)* identifying and quantifying the zwitterionic pairs onto the bioceramic surface; *iii)* revealing the chemical bonds that maintain organic functions linked to the bioceramic surface; *iv)* determining the homogeneity degree regarding zwitterionic moieties distribution onto the materials surface; and *v)* predicting the ionization status of zwitterionic pairs at physiological conditions depending on the surface electrochemistry of these matrices. There are a number of techniques that satisfy some of these conditions, such as Fourier transform infrared spectroscopy (FTIR), Scanning transmission electron microscopy (STEM) and energy dispersive X-ray spectroscopy (EDS), X-ray photoelectron spectroscopy (XPS), solid-state nuclear magnetic resonance (NMR) spectroscopy and zeta(ζ)-potential (**Figure 3**), but is their overall combination which give complete information about zwitterionic bioceramics.

4.1. Fourier Transform Infrared Spectroscopy

FTIR is a powerful qualitative tool to identify the presence of functional groups either charged or neutral, (*e.g.* $-NH_3^{\oplus}/NH_2$ and $COO^{\ominus}$/COOH pairs), which allows determining the zwitterionic nature of a bioceramic [17,24,31,32]. Figure 3 shows FTIR spectra corresponding to pure silica SBA-15 and zwitter-SBA15 materials, chosen as representative examples (structure **4**, Figure 2) [31]. FTIR spectrum of zwitter-SBA15

sample displays bands at 3344 and 1500 cm^{-1} corresponding to the NH stretching and deformation frequencies, and bands at 3100 and 590 cm^{-1} corresponding to $–NH_3^{\oplus}$ stretching and deformation frequencies, respectively. On the other hand, FTIR spectrum also shows bands at 1620 and 1400 cm^{-1} of the anti-symmetric and symmetric frequencies of $COO^{\ominus}$ and a band at 1720 cm^{-1} of COOH group. Hence, the presence of $–NH_3^{\oplus}$/ $COO^{\ominus}$ pairs is shown, confirming the zwitterionic nature of this bioceramic.

4.2. Scanning Transmission Electron Microscopy

STEM technique give information related to the overall distribution of the functional groups into the bioceramic surface. This is very important to determine the degree of homogeneity of the *zwitterionization* process throughout the sample. For instance, EDS coupled to STEM was used to study zwitter-SBA15 mesoporous crystals (structure **5**, Figure 2) [32]. The aim was investigating the distribution of the functional groups into the mesoporous crystals. EDS study was carried out by selecting different mesoporous crystals and recording N, O and Si maps. Mapping of the square region highlighted in the STEM image (Figure 3) showed a very weak N signal, suggesting that N from DAMO were randomly and homogeneously distributed within the silica framework.

4.3. X-ray Photoelectron Spectroscopy

XPS is a powerful technique to characterize zwitterionic surfaces due to its capability to identify the surface groups, the chemical state of the atoms, and the relative abundance in the different surfaces with high rate of sensibility [31,45,46]. For instance, XPS has been used to characterize zwitter-SBA15 bioceramic (structure **4**, Figure 2) [31]. Bare SBA-15 and zwitter-SBA15 showed binding energies of O1s and Si2p core-levels at 532.9 and 103.4 eV, respectively, which are characteristic of silica-based materials. In the case of zwitter-SBA15 sample the C1s line profile was fitted to three components at

284.9, 286.8, and 288.6 eV (Figure 3). The component at 284.9 eV is associated with CC/CH bonds of the alkyl chains of APTES; the component at 286.6 eV can be attributed to C-O moieties of remaining surfactant; and finally at the component located at 288.6, which represents 10% of the total area of the peaks, is characteristic of COO functional groups. The high resolution N1s line profile was rather asymmetrical displaying broadening in the high binding energy side, suggesting that more than one component was present. In fact, the N1s peak was satisfactorily fitted to two components at binding energies of 400.9 and 402.9 eV, which are usually assigned to -NH_2 and $–NH_3^{\oplus}$ moieties, respectively (Figure 3). Moreover, from peak areas and atomic sensitivity factors it was possible to quantitatively evaluate the species present onto the bioceramic surface.

4.4. Solid-State Nuclear Magnetic Resonance Spectroscopy

Solid state NMR spectroscopy is a powerful technique to assess the chemical grafting of organic groups containing zwitterionic moieties to the surface of bioceramics bearing –OH groups, such as –SiOH and –POH by using ^{29}Si NMR and ^{31}P NMR probes, respectively. For example, ^{29}Si solid state NMR was used to study the nature of the chemical bonds between organic functions and silica matrix in zwitter-SBA15 bioceramic (structure **4**, Figure 2) [31]. These studies revealed the appearance of signals attributable to T^n units [R-Si$(OSi)_n$- $(OX)_{3-n}$] (X = H,C) of the organosilane groups grafted onto the silica matrix. The highest relative abundance corresponded to Si atoms in T^3 environments [R-Si$(OSi)_3$] (signal at *ca.* -70 ppm), which confirmed the existence of covalent linkages between the silica surface and the organic groups (Figure 3).

In another example, ^{31}P NMR spectroscopy was used to characterize the covalent grafting of organosilanes (APTES and CES) onto the surface of HA [17]. ^{31}P NMR spectra of both HA and zwitter-HA displayed a narrow resonance at 1.8 ppm (q^0)

assigned to orthophosphate tetrahedron [PO_4] species characteristic of HA (Figure 3). However, ^{31}P NMR spectrum of zwitter-HA exhibited an additional weak signal at *ca.* 8 ppm, which fall within the range typically found for ^{31}P in q^1 tetrahedra. This finding evidenced the formation of P–O–Si bonds and confirmed the successful silylation of HA surface by using suitable alkoxysilanes.

Finally, it is well-known that solid state ^{13}C NMR spectroscopy can be also used to confirm the efficient surface functionalization of bioceramics (data not shown).

4.5. Zeta (ζ)-potential

When zwitterionic bioceramics are intended for given biomedical applications it is essential to determine the pH values where the zwitterionic nature of these bioceramics is preserved. For this purpose, the isoelectric point (IEP) of samples in aqueous media, which match the zero charge point, is usually determined. Thus, ζ–potential measurements of bioceramic suspensions in aqueous medium at different pH values are recorded. Figure 3 displays, as a representative example, ζ-potential *vs* pH plots for SBA-15 and zwitter-SBA15 (structure **5**, Figure 2) (Figure 3) [32]. The graph clearly reveals the preservation of the zwitterionic nature of zwitter-SBA15 at the physiological pH of 7.4 (ζ-potential = 0), which accounts for the potential biomedical application of this bioceramic.

5. Potential Biomedical Applications of Zwitterionic Ceramics

This section focuses on zwitterionic bioceramics with potential biomedical applications in local drug delivery, bone tissue regeneration and nanomedicine. Table 1 summarizes the different bioceramics reported to date, the type of functionalization used to provide them of zwitterionic nature, their non-fouling and/or antibacterial properties, their development phase and their potential biomedical applications.

5.1. Local Drug Delivery

The treatment of bone diseases by local drug delivery from implantable bioceramics has several advantages compared to systemic administration, such as minimized side effects and risk of overdose, as well as improved bioavailability and effective drug concentrations at the target site [1]. Among bioceramics SMMs have been widely explored as local drug delivery systems [47-50]. These matrices exhibit excellent properties, such as high surface areas and pore volumes, tunable and narrow pore size distributions and easily functionalizable surfaces, which make them ideal matrices to host diverse biologically active molecules [51]. The relatively easy functionalization of their surface permits SMMs being submitted to optimized *zwitterionization* procedures, which represents an added value for their potential biomedical applications. Figure 4 displays an example of a SBA-15 type mesoporous bioceramic bearing $–NH_3^{\oplus}/–SiO^{\ominus}$ and $>NH_2^{\oplus}/–SiO^{\ominus}$ pairs [32] (structure **5**, Figure 2), whose zwitterionic nature was preserved at pH 7.4. *In vitro* bacterial adhesion assays indicated a 99.9% reduction in *S. aureus* bacterial adhesion in zwitter-SBA15 compared to pristine SBA-15. In this case the bacterial adhesion was determined both qualitatively and quantitatively, by confocal microscopy studies and the counting of colony forming units (CFUs) [52], respectively. Moreover, zwitter-SBA15 was capable to host antibiotics and release them in a sustained fashion. Thus, *in vitro* loading and release assays using cephalexin as a model antibiotic proved that zwitter-SBA15 can host *ca.* 13 mg g^{-1} of drug and release for more than 15 days. This dual role, antibacterial adhesion and antibiotic drug delivery, constitutes a step forward for development of advanced bioceramics for the management of bone implant infection.

When aimed at developing implantable bioceramics it would be desirable to attain surfaces able to inhibit bacterial colonization whereas permitting the adhesion of tissue

cells. Thus, our research group developed a zwitterionic SBA-15 containing $–NH_3^{\oplus}$/$–COO^{\ominus}$ surface moieties (Figure 5.A) [31] (structure **4**, Figure 2). This zwitter-SBA15 exhibited zwitterionic nature at pH values ca. 5.5, whereas the surface was negatively charged at the physiological pH of 7.4 (Figure 5.A). The ability of zwitter-SBA15 to inhibit *E. coli* adhesion was *in vitro* evaluated at pH 5.5, which is representative of severe inflammation/infection conditions [16]. The obtained results indicated that zwitter-SBA15 reduced bacterial adhesion *ca.* 93% relative to bare SBA-15 (Figure 5.B), as derived from the counting of CFUs. Nonetheless further studies are needed with other pathogens, such as *S. aureus* or *S. epidermidis* to better define the antibacterial properties of these materials. In addition, *in vitro* tests with human Saos-2 osteoblasts cultures allowed investigating the cell response onto zwitter-SBA15 at the physiological pH of 7.4. The results showed that zwitter-SBA15 exhibited good biocompatibility, with Saos-2 osteoblasts adhering, proliferating and preserving their morphological and functional features (Figure 5.C) [16].

5.2. Bone Tissue Regeneration

The recent progresses in the design of advanced bioceramics with tailored surface properties open promising insights in the development of novel biocompatible implants with antibacterial adhesion for bone tissue regeneration. In this sense HA is one of the bioceramics more widely exploited in clinic [53]. HA is a calcium phosphate-based widely used in many areas of dental and orthopedic reconstructive medicine due to its similarity with natural bone, biocompatibility, bioactivity and osteoconductivity [53,54]. HA is commercially available in several physical forms, including powders, particles, granules, dense blocks, cements, porous three-dimensional (3D) scaffolds, implant coatings and composite components. Providing HA of antibacterial adhesion capability would add an interesting property to this bioceramic for potential clinical

purposes. Thus, our research group provided the surface of HA of zwitterionic nature by incorporating $-NH_3^{\oplus}$ /$-COO^{\ominus}$ pairs by post-synthesis functionalization with APTES and CES (Figure 6) [17]. In a first approach, the *zwitterionization* method was optimized in HA powders prepared by the controlled crystallization method. ζ-potential studies indicated that the zwitterionic nature of zwitter-HA was preserved at the physiological pH of 7.4. Then, this methodology was successfully applied to HA shaped as 3D-scaffolds prepared by using rapid prototyping. This constitutes a major advance in bone tissue regeneration technologies, since it is possible to functionalize 3D-shaped macroporous pieces using a reproducible, easy and cost-efficient methodology. *In vitro E. coli* adhesion assays at physiological conditions indicated that zwitter-HA was able to reduce bacterial adhesion in a 99% compared to pristine HA (Figure 6), as derived from the counting of CFUs. Furthermore, the biocompatibility of zwitter-HA was evaluated using HOS osteoblast-like cell cultures. SEM micrographs showed viable and well-spread cells, which have preserved their typical osteoblast morphology, exhibiting polygonal shapes with small filopodia-like projections anchored to both surfaces (Figure 6). Regarding cell morphology, no differences were observed between zwitterionic and bare HA samples. The study of the cell colonization of the different levels of 3D scaffolds proved that the different scales of porosity, *i.e.* channels of *ca.* 800 μm and macropores at 0.01-600 μm range, allowed good cellular internalization with adequate cell anchorage and subsequent cell colonization over the entire surface of the scaffolds.

5.3. Nanomedicine

Nanoparticles (NPs) usually refer, but are not limited, to particles ≤ 100 nm in diameter. They exhibit physical and chemical characteristics different from those of bulk

materials, opening many possibilities to solve different problems especially in the nanomedicine arena [55-58]. There are two main reasons that support this fact*: i)* the intrinsic features of NPs can be appropriately exploited for therapy and/or diagnosis purposes; and *ii)* the NPs surface can be functionalized with a great variety of species to incorporate targeting ligands, modulate drug adsorption and pharmacokinetics and/or to permit extra ways of therapy and/or detection. Inorganic NPs exhibit noteworthy advantages compared to their organic counterparts, such as higher thermal, chemical and mechanical stabilities under physiological conditions. They also show good biocompatibility and relatively low degradation rates. Herein we focus on ceramic NPs, highlighting silica NPs and SPIONPs, whose synthesis, features and biomedical applications have been extensively overviewed [59-64]. SPIONPs have potential application in magnetic resonance image (MRI), hyperthermia treatment and even iron deficient anemia in adults. Whatever the application, the size, shape and surface properties of NPs have enormous effect on their biodistribution and pharmacokinetics *in vivo* [65,66]. As above discussed, one of the major limitations that NPs have to face once *in vivo* administered is *opsonization*, which adds complexity to their design and manufacture for biomedical applications. A widely used approach to overcome this concern consists in attaching PEG to the surface of NPs [67]. *Zwitterionization* has emerged as a powerful alternative to reduce the rate and degree of nonspecific proteins adsorption to the NPs surface and therefore inhibiting the formation of a "protein corona". In this section we overview some biomedical applications of zwitterionic ceramic NPs.

5.3.1. Zwitterionic Silica Nanoparticles

Silica-based NPs have been widely proposed for application in nanomedicine [60,68]. Pure silica NPs can be acquired from commercial homes, whereas those incorporating

fluorescent dyes, chemotherapeutic agents and targeting ligands can be prepared in the lab. It is worth of mention the possibility to functionalize the silica surface with diverse organic groups via silane chemistry [69-71]. Silica surfaces are frequently used as the outer layer in different core-shell NPs. Their physical chemical properties and interaction with biological fluids have been broadly investigated. Their extensive use and versatility make silica NPs outstanding model system to develop and evaluate new nanoparticle surface chemistry such as *zwitterionization*.

Varied *zwitterionization* strategies have been set up to counter the unspecific protein adsorption onto the surface of NPs. Rosen *et al.* grafted Cys amino acid to LUDOX^{TM} AS-40 (40% wt colloidal silica in water) [24] (structure **1**, Figure 2). Zwitter-NPs exhibited excellent stability in both salt and single "sticky" protein solutions of lysozyme (Lys, positively charged), bovine serum albumin (BSA, negatively charged), and human serum. The surface of Cys-coated particles bears a much smaller charge at physiological pH and has the added advantage of being strongly hydrated due to its zwitterionic character. These characteristics make the surface largely resistant to fouling by serum proteins, which will tend to stick to highly charged surfaces.

Estephan *et al.* linked short-chain zwitterionic SB siloxane (SBS) derivatives (structure **2**, Figure 2) onto the surface of LUDOX^{TM} AS-40 without evident increase in the hydrodynamic size [26,27]. The resulting zwitter-NPs were stable against aggregation in 3M NaCl and 50% (v/v) fetal bovine serum (FBS).

As previously commented, a widely exploited strategy consists in the coating of silica NPs with zwitterionic polymers such as pSB, pCB, and pAA derivatives. For instance, Dong *et al.* grafted pSBMA (structure **6**, Figure 2) onto silica NPs via SI-ATRP to attain zwitter-NPs of *ca.* 120 nm in size and a ζ-potential of -7.5 mV under salt-free conditions.[39] The slightly negative surface charge was ascribed to

incomplete coverage of the negatively-charged silica surface (structure **6**, Figure 2). The phase behavior of these zwitter-NPs *versus* temperature in aqueous solutions and their stability in protein phosphate buffer saline (PBS) solutions were evaluated. These NPs exhibited and upper critical solution temperature in aqueous solution that can be controlled by varying the pSBMA molecular weight, ionic strength, zwitter-NPs concentration and solvent polarity. Zwitter-NPs were stable for at least 72 h in both Lys and BSA solutions as well as PBS. The adjustable phase behavior and stability in protein solutions, combined with the reported biocompatibility and hemocompatibility of zwitterionic polymers make these zwitter-NPs good candidates for application in biosensing, smart drug delivery, implanted devices and other biotechnologies. Sun *et al.* described the attachment of a zwitterionic pSB derivative (poly(2-(dimethylamino)-co-3-dimethyl-(metacryloyloxyethyl)ammonium propanesulfonate) to the outermost surface of MSNPs by RAFT polymerization, affording MSN-p(DMAEMA-co-DMAPS) (zwitter-MSNPs) [42]. pDMAPS conferred the nanosystem of biocompatibility and pDMAEMA made the polymer shell temperature-responsive. Rhodamine B (RhB), chosen as model molecule, was loaded into the pores of zwitter-MSNPs and dye release was monitored by varying the temperature in saline solution. Heating provoked volume contraction of polymer shells, which accelerated drug release. Besides, *in vitro* assays demonstrated that zwitter-MSNs had minimal cytotoxicity on HeLa cells at 200 μg mL^{-1}. These findings open promising expectations in cancer treatment through the combination of thermal and chemotherapy. pCB derivatives have been also linked to the surface of silica NPs [40,41]. For instance, Jiang and coworkers grafted biocompatible zwitterionic poly[(3-acryloylamino)propyl](2-carboxyethyl)-dimethylammonium] (pCBAA) to silica NPs via SI-ATRP [41]. Zwitter-NPs exhibiting two different polymer thicknesses were stable for at least 72 h in both Lys and BSA

protein solutions, evidencing the good capability of pCBAA layers to enhance biointerfacial characteristics of silica NPs.

5.3.2. Superparamagnetic Iron Oxide Nanoparticles

SPIONPs exhibit distinctive features, such as high saturation magnetization, high chemical stability and potentially reduced toxicity, which make them attractive contrast agents to enhance the *in vitro* and *in vivo* magnetic resonance imaging (MRI) [72]. The preparation of uniformly sized SPIONPs usually involves the use of organic solvents [73], and their modification by ligand exchange is essential to provide NPs of hydrophilic properties that allow them being considered for biomedical applications [74]. Zwitterionization of SPIONPs has arisen as a powerful tool to improve their potential in nanomedicine. Among zwitterionic ligands, SB derivatives have been widely exploited to enhance the stability of SPIONPs [28-30,75]. Thus, Stephan *et al.* prepared stable aqueous dispersions of SPIONPs in one step in the presence of a zwitterionic SBS derivative as the stabilizing/capping/solubilizing ligand (structure **2**, Figure 2). The hydrodynamic diameter of the NPs was tailored by setting up the concentration of zwitterion siloxane, which eventually yielded monodisperse NPs with small enough diameters for renal clearance (<6 nm). Zwitter-NPs were properly dispersed and stable in aqueous media in the pH range 6−9, but aggregated at lower pH, probably due to the existence of some uncapped pH-sensitive Fe-OH groups on the surface of NPs. Nonetheless, zwitter-NPs showed lower magnetization values than non-zwitterionic-NPs due to amorphous content and spin canting, characteristic for NPs of such size. NPs showed minimal non-specific adsorption of proteins, which suggest the materials will circulate well *in vivo*.

Bawendi and coworkers developed zwitterionic DOPA sulfonate (ZDS) ligand coated SPIONPs (structure **3**, Figure 2) for use in various medical applications [28,29] ZDS-coated SPIONPs were characterized by small hydrodynamic diameters, low non-specific protein adsorption, the possibility of specific labeling, and stability with respect to time, pH, and salinity. Importantly, ZDS coatings did not reduce superparamagnetism or saturation magnetization of as-synthesized hydrophobic SPIONPs. Moreover, ZDS-coated SPIONPs showed only small non-specific uptake into HeLa cancer cells *in vitro* and low non-specific binding to serum proteins *in vivo* in mice. Zhou *et al.* also used ZDS to coat 4.8 nm-sized gadolinium-embedded iron oxide (GdIO) NPs to produce T1 contrast agents with a hydrodynamic size of 5.2 that were stable in PBS and 20% FBS [30]. *In vivo* studies revealed high GdIO NPs accumulation in the kidneys and bladder after 10 min of injection, whereas NPs were found in the urine after 4 h of injection, indicating that they experience fast clearance via glomerular filtration and excretion process. Even so, the half-time life of NPs in the bloodstream (around 50 min) was enough to permit their passive accumulation within a subcutaneous ovarian cancer.

Zwitterionic polymers have been also used to coat SPIONPs. For example, Jiang and coworkeers prepared pCBMA-DOPA-coated SPIONPs (structure **7**, Figure 2), which were stable in salt solution and undiluted human blood serum, and exhibited ultra-low uptake by macrophages [43]. PAA-DEAPA-coated SPIONPs were developed by Gu and coworkers (structure **8**, Figure 2) [44].This strategy provided a near-zero ζ–potential within the pH range of 7.5-8.5 and good anti-fouling properties, as proved by a noticeable improved blood circulation time in mice, implying minimized murine macrophage uptake. *In vivo* tests using MRI evidenced that zwitterionic PAA-DEAPA-NPs could provide good blood pool image contrast up to 2 h after injection, whereas PAA-coated NPs were scarcely detected in the bloodstream after 20 min post-injection.

Conclusion

Zwitterionization constitutes an emerging tool for the design of advanced bioceramics for biomedical applications. The possibility to tailor the physicochemical properties of bioceramics at the nanoscale to inhibit bacterial and non-specific protein adhesion opens up new gates to give solutions to major clinical issues, such as bone implant infection and the lack of efficiency of therapy and diagnosis with nanocarriers.

However there are also some challenges that have to be faced before the entrance of zwitterionic bioceramics into advanced development clinical phases. They include the preservation of biocompatibility, suitable pharmacokinetics and bone healing capability in bioceramics for local drug delivery, bone tissue regeneration and nanomedicine. We are in the beginning of a very exciting scientific journey and much research effort is needed before moving from the lab bench to the bedside.

Acknowledgments

Authors acknowledge Ministerio de Economía y Competitividad (MINECO), Spain, for funding through projects MAT2012-35556 and CSO2010-11384-E (Agening Network of Excellence).

Conflicts of Interest

The authors declare no conflict of interest.

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Tables

Table 1. Sumary of different zwitterionic bioceramics, their functionalisation strategies, properties, cell *in vitro* assays and potential biomedical applications.

Bioceramic[a]	Functionalisation	Properties[b]		Cell *in vitro* assays[c]	Potential Biomedical Applications	Refs.
		Non-fouling	Antibacterial			
SMMs	Structure 4	STI	*E. coli*	Saos-2	Local drug delivery	[16,31]
	Structure 5	–	*S. aureus*	–		[32]
HA	Structure 4	–	*E. coli*	HOS	Bone tissue regeneration	[17]
Silica NPs	Structure 1	Lys, BSA, HS	–	–	Nanomedicine (drug delivery)	[24]
	Structure 2	–		–		[25,26]
	Structure 6 and derivatives	Lys, BSA		HeLa		[39,42]
	Structure 7 and derivatives	Lys, BSA		–		[40,41]
IONPs	Structure 2	BSA, FBS	–	–	Nanomedicine (imaging, hyperthermia)	[27]
	Structure 3	FBS	–	HeLa **	Nanomedicine (imaging, sensing, hyperthermia)	[28-30]
	Structure 6	BSA	–	–	Nanomedicine (imaging)	[75]
	Structure 7	HBS	–	HeLa RAW 264.7 HUVEC	Nanomedicine (imaging)	[43]
	Structure 8	HS	–	RAW 264.7 ***	Nanomedicine (imaging)	[44]

[a] SMMs: Silica-Based Mesoporous Materials; HA: hydroxyapatite; Silica NPs: Silica Nanoparticles; IONPs: Iron Oxide Nanoparticles.

[b] STI: Soybean Trypsin Inhibitor; Lys: Lysozime; BSA: Bovine Serum Albumin; HS: Human Serum, HBS: Human Blood Serum.

[c] Saos-2: Human osteosarcoma cell line; HOS: Human osteosarcoma cell line; HeLa: Human epitheloid cervix carcinoma cell line; HUVEC: Human umbilical vein endothelial cells; RAW 264.7: mouse macrophague cell line; (******) *In vivo* assays in mice have been also performed; (***) *In vivo* assays in rabbits have been also performed.

Figures

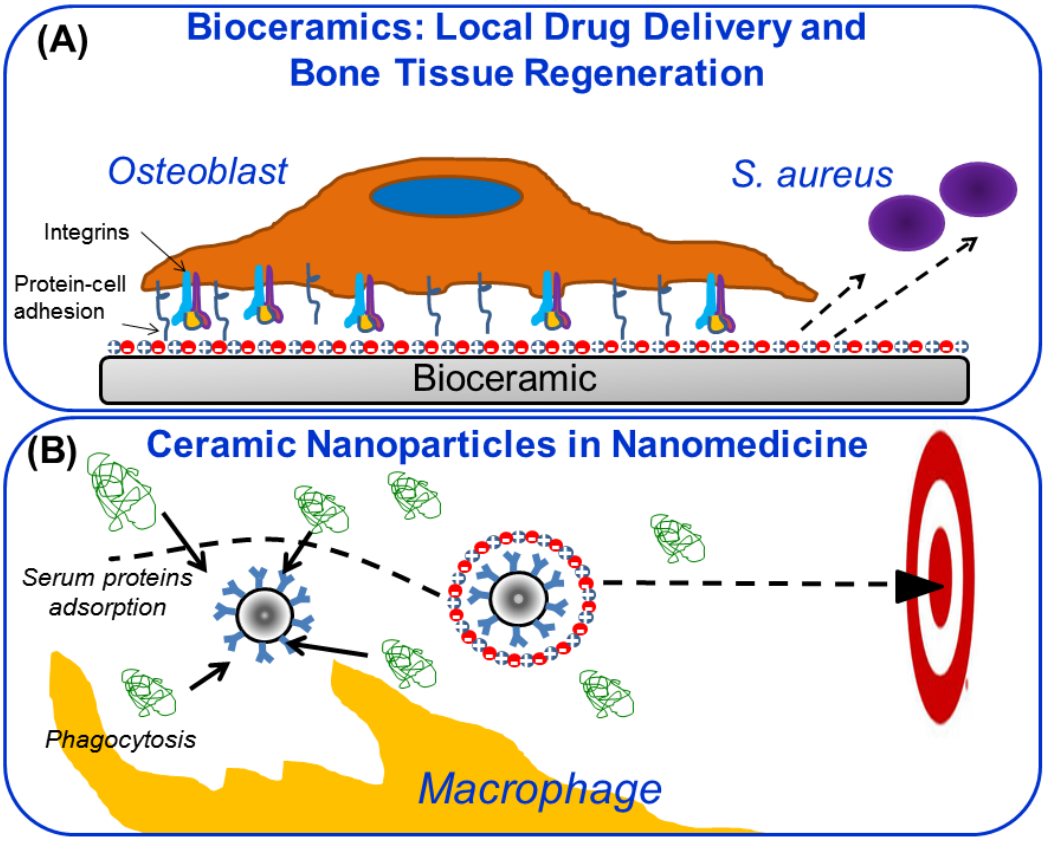


Figure 1. **A)** Cell and bacteria interaction into bioceramics for drug delivery systems and bone tissue regeneration. The scheme shows the eukaryotic cells interact with a bioceramic surfaces through of discrete attachment points (integrins). On the contrary the scheme also schematizes bacteria with smaller size and stiffness compared to eukaryotic cell, showing the main interaction based on electrostatic attraction forces between bacteria and bioceramic surfaces. **B)** Schematic representation of zwitterionic-coated nanoparticle and its resistance to non-specific protein adsortion.

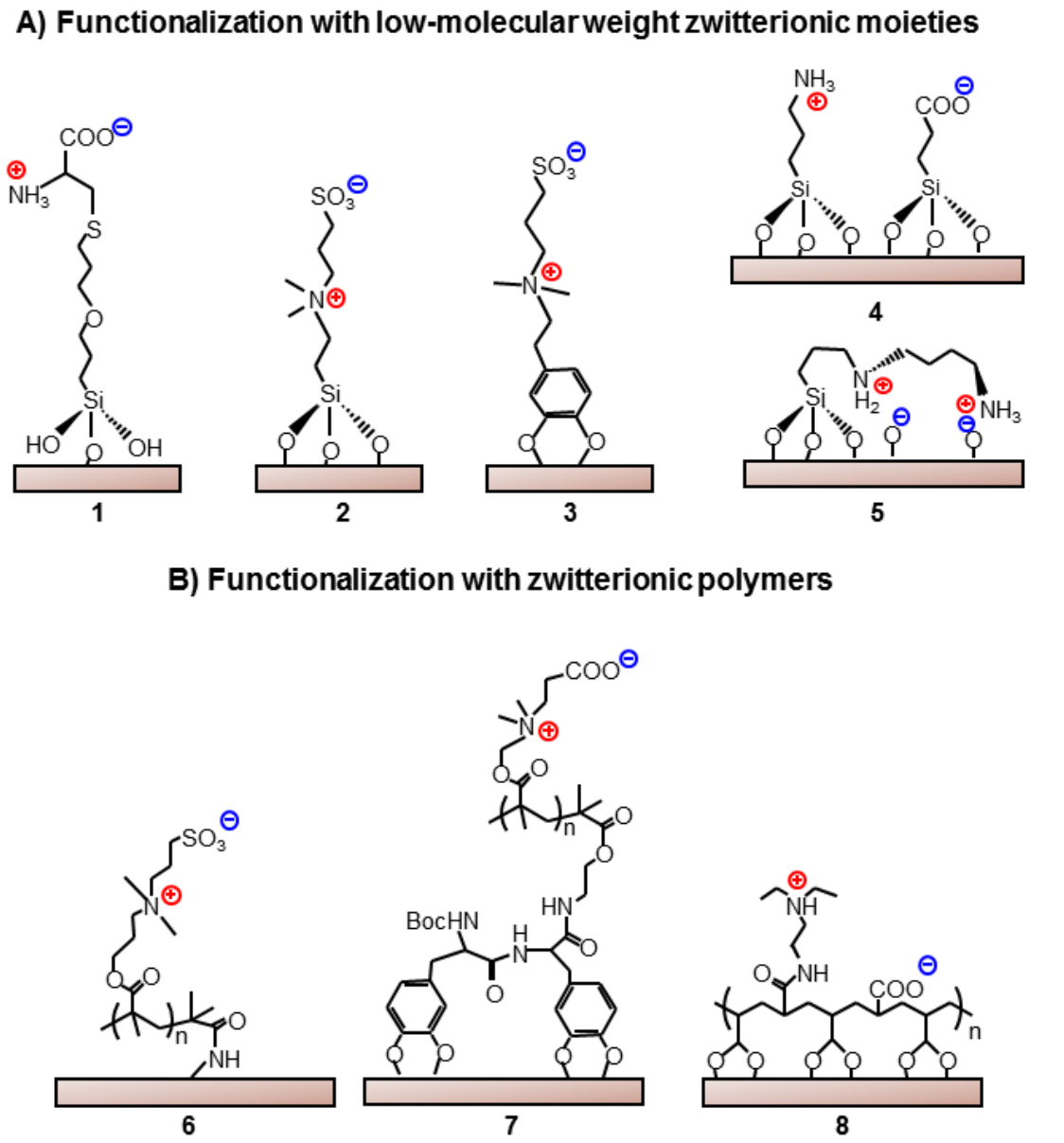


Figure 2. Chemical strategies for the *zwitterionization* of bioceramics. **A)** *Grafting of low-weight zwitterionic moieties*: **1** Cysteine derivatives; **2** Sulfobetaine siloxane derivatives; **3** Dopamine sulfonate ligands; **4** 3-aminopropyltrimethoxysilane (APTES) and carboxyethylsilanetriol sodium salt (CES); **5** (N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane) (DAMO). **B)** *Grafting of zwitterionic polymers:* **6** Poly(sulfobetaine methacrylate) (pSBMA); **7** Poly(carboxybetaine methacrylate) (pCBMA) bearing 3,4-dihydroxyphenyl-1-alanine (DOPA) moieties; **8** 3-(diethylamino)propylamine (DEAPA) coupled to poly(acrylic acid) (PAA).

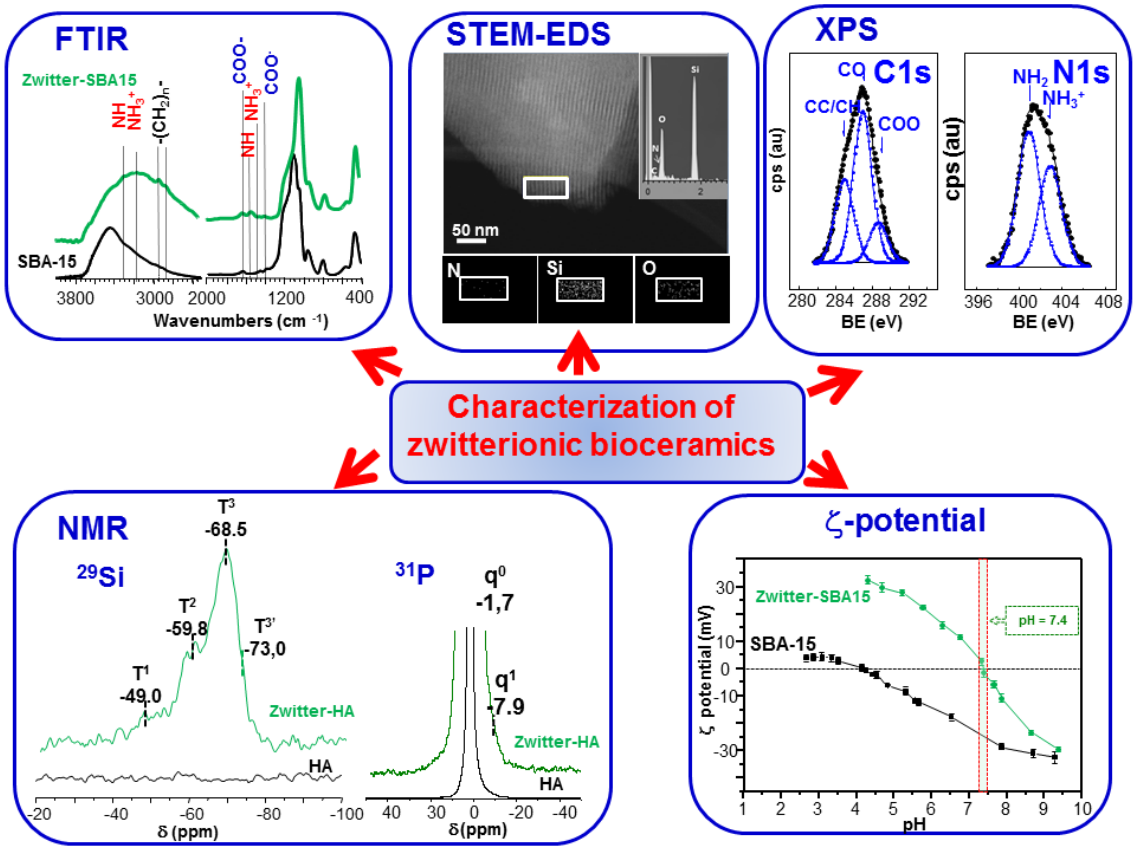


Figure 3. Schematic representation of the main techniques used for the characterization of zwitterionic bioceramic surfaces.

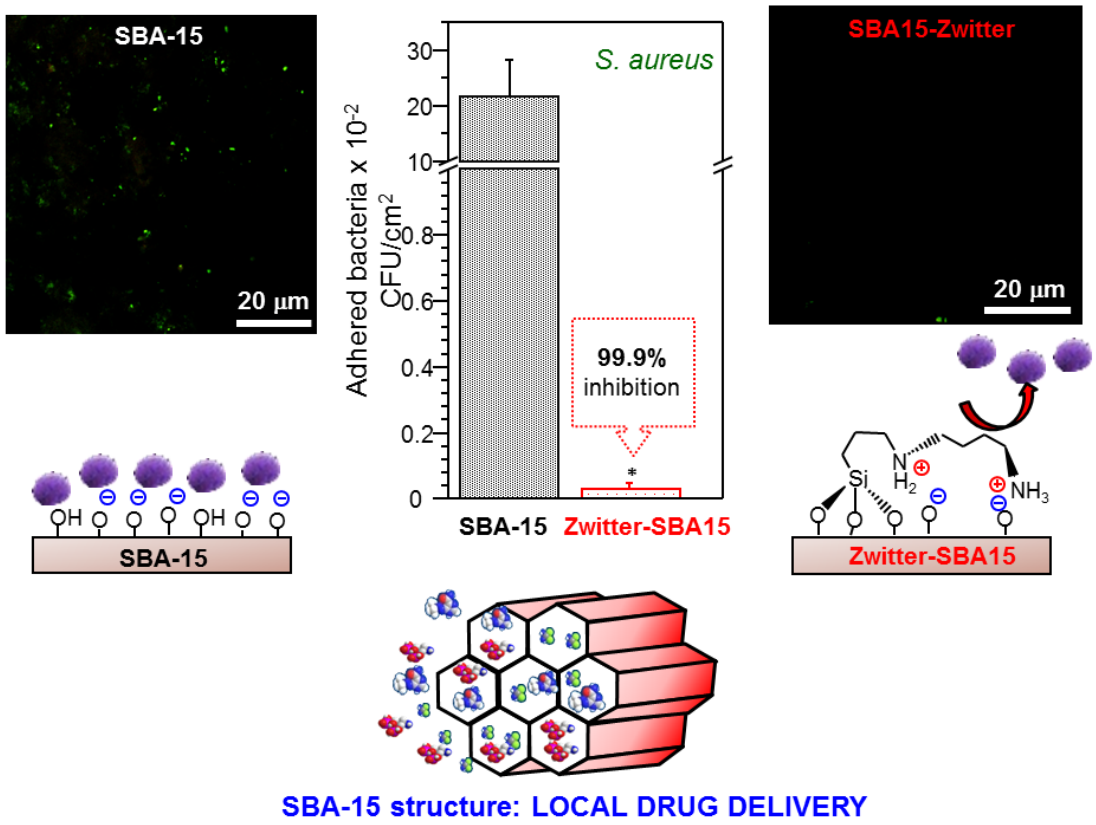


Figure 4. SBA-15 structures as local drug delivery systems. Schematic depiction of pure silica SBA-15 and zwitterionic SBA-15 prepared following the co-condensation route in the presence of N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane (DAMO) (SBA15-Zwitter). Counting of colony forming units of *S. aureus* after 90 of culture onto SBA-15 and SBA15-Zwitter surfaces. Statistical significance: **p* <0.01. Confocal fluorescence micrographs of adhered S. aureus onto SBA-15 and SBA15-Zwitter surfaces after staining with Baclight® Kit™ [32].

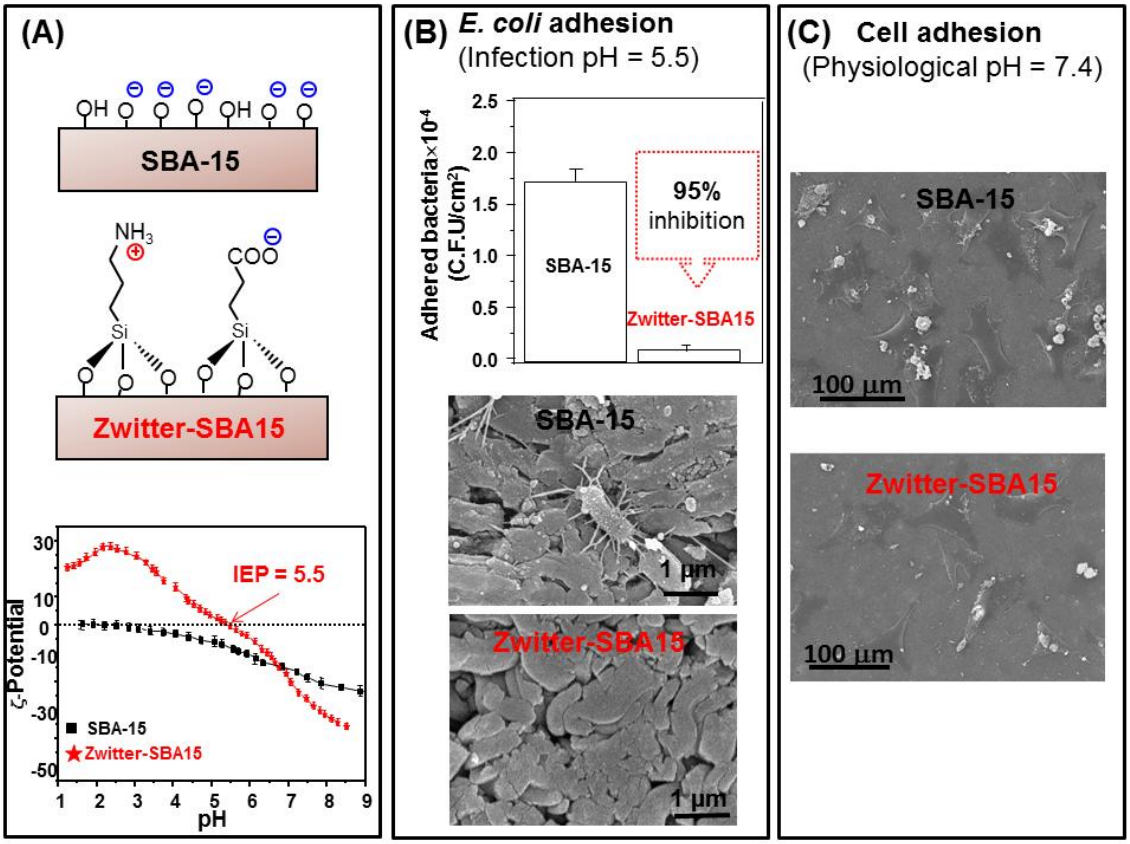


Figure 5. **A)** Schematic representation of SBA-15 mesoporous bioceramic before and after bifunctionalization by co-condensation route with APTES and CES organosilanes moeties (structure 4, Figure 2). ζ-potential measurements showing that zwitter-SBA15 exhibit s zwitterionic nanture at pH values of 5.5, whereas the surfaces is negatively charged at pH values of 7.4 (physiological condictions). **B)** In vitro E.coli adhesion assays after 90 min of incubation indicating a notable reduce of bacteria adhesion in zwitter-SBA15 surfaces at pH 5.5. SEM micrographs showing in details the bacteria attached to the surfaces of pristine SBA-15 surface and the absence of bacteria onto zwitter-SBA15. **C)** *In vitro* osteoblast culture assays in physiological condition, showing in detail similar osteoblast cell spreading onto both surfaces [16].

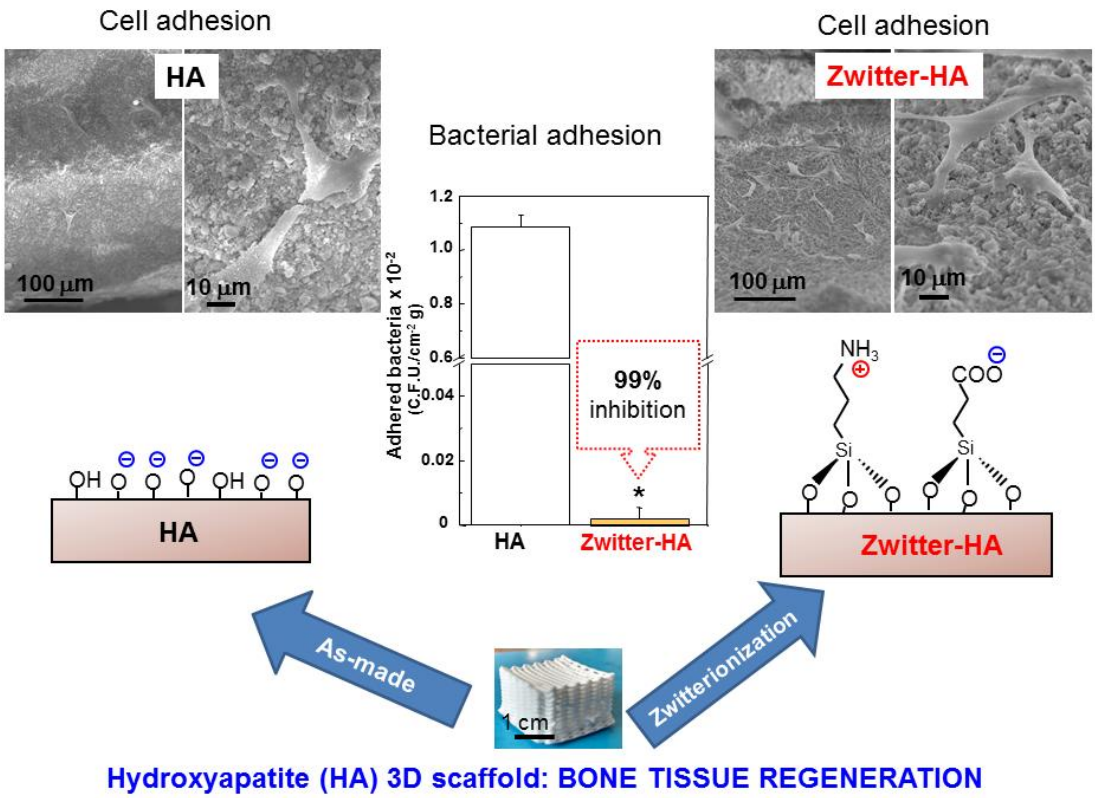
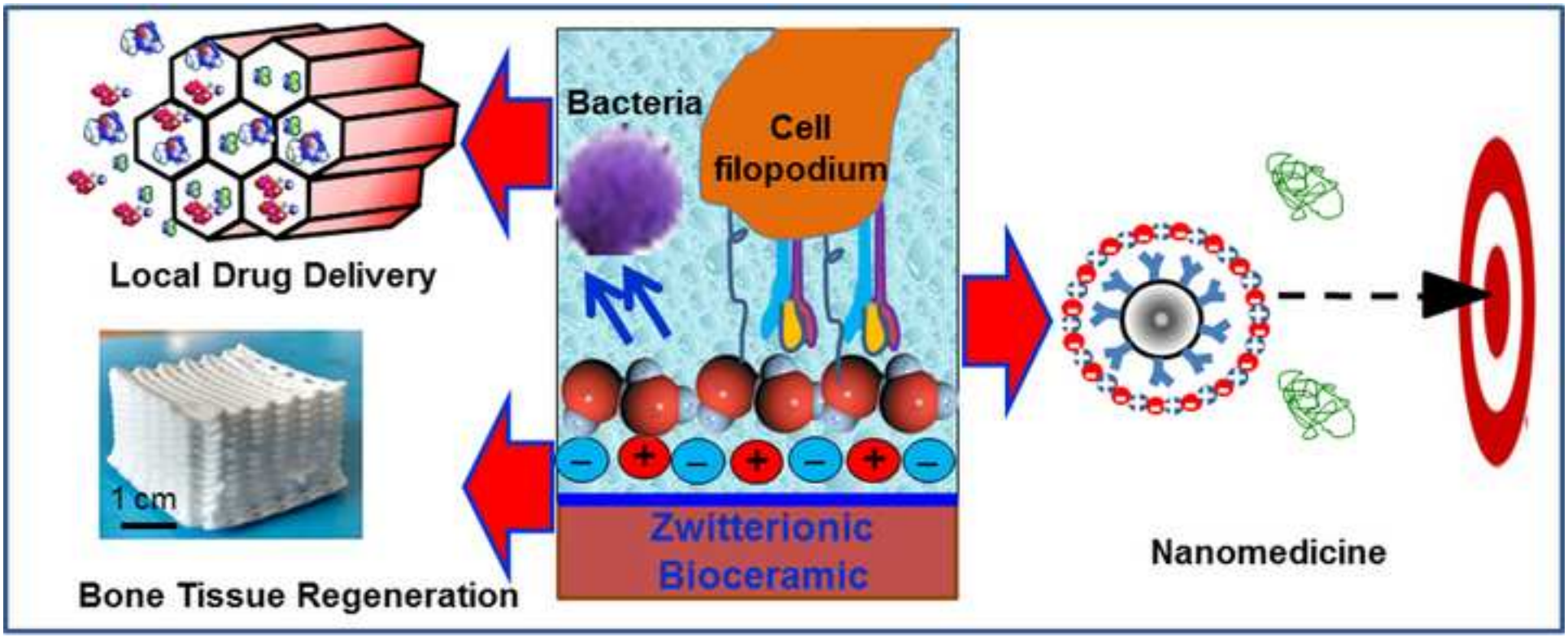


Figure 6. Schematic representation of the surface of hydroxyapatite (HA) 3D scaffolds, before (HA) and after postsynthesis zwitterionization with 3-aminopropyltrimethoxysilane (APTES) and carboxyethylsilanetriol sodium salt (CES) (HA-Zwitter). *E. coli* adhesion attachment to HA and HA-Zwitter surfaces. Statistical significance: **p* <0.01. SEM micrographs at 200 x and 1000 x magnifications of the surfaces of HA and HA-Zwitter scaffolds after 24 hours of cells spreading assays with HOS osteoblast culture [17].

*Graphical Abstract



***Statement of Significance**

Zwitterionic bioceramics are receiving growing attention by the biomaterials scientific community due to their great potential in bone tissue regeneration, local drug delivery and nanomedicines. Herein, the different strategies developed so far to synthetize and characterize zwitterionic bioceramics with potential clinical applications are summarized.

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Madrid, January 27th 2016

Dear Prof. Marc Bohner and William R. Wagner,

Thank you very much for your kind e-mail concerning the Manuscript **"Zwitterionic Ceramics for Biomedical Applications"** authored by *Isabel Izquierdo-Barba, Montserrat Colilla and María Vallet-Regí* (REF: AB-15-2236), which you are considering for publication in the Special Issue on Zwitterionic Materials in Acta Biomaterialia.

We sincerely appreciate editor's and reviewers' remarks to improve the article and, accordingly, we have modified our manuscript following their recommendations. The modifications are highlighted in the manuscript and detailed responses to the reviewers' comments are included bellow.

We hope that the revised version of our manuscript is now appropriate for publication in International Acta Biomaterialia.

Thank you for your kind consideration of our work.

Sincerely,

Prof. María Vallet-Regí.